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Occurrence of Two Different Forms of Protocatechuate 3,4-Dioxygenase in a *Moraxella* sp.

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Two alternative forms of protocatechuate 3,4-dioxygenase (PCase) have been purified from *Moraxella* sp. strain GU2, a bacterium that is able to grow on guaiacol or various other phenolic compounds as the sole source of carbon and energy. One of these forms (PCase-P) was induced by protocatechuate and had an apparent molecular weight of 220,000. The second form (PCase-G) was induced by guaiacol or other phenolic compounds, such as 2-ethoxyphenol or 4-hydroxybenzoate. It appeared to be smaller (M_r 158,000), and its turnover number was about double that of the former enzyme. Both dioxygenases had similar properties and were built from the association of equal amounts of nonidentical subunits, α and β, which were estimated to have molecular weights of 29,500 and 25,500, respectively. The $(\alpha\beta)_3$ and $(\alpha\beta)_4$ structures were suggested for PCases G and P, respectively. On the basis of two-dimensional gel electrophoresis, the α and β polypeptides of PCase-G differed from those of PCase-P. Amino acid analysis supported this conclusion. Both PCases, however, had several other properties in common. It is proposed that both isoenzymes were generated from different sets of α and β subunits, and the significance of these data is discussed.

Protocatechuate (3,4-dihydroxybenzoate) is a frequent intermediate in the biodegradation of many aromatic compounds by bacteria. The non-heme iron enzymes protocatechuate dioxygenases couple O_2 bond cleavage with ring fission of protocatechuate, either between the two phenolic groups (the so-called ortho cleavage) or beside one of these (the meta cleavage type). Protocatechuate 3,4-dioxygenase (PCase, EC 1.13.1.3) is a ferric enzyme catalyzing the ortho cleavage of protocatechuate to yield β-carboxy-*cis*,*cis*-muconate, the first intermediate in the pathway leading to 3-oxoadipate (7, 31). PCase has been characterized in a number of organisms, including *Pseudomonas putida* (3, 7, 25, 26), *P. cepacia* (19), *P. aeruginosa* (11), *Acinetobacter calcoaceticus* (13), *Azotobacter vinelandii* (10), *Brevibacterium fuscum* (34), *Nocardia erythropolis* (17), *Rhizobium trifolii* (6), other *Rhizobium* spp., *Agrobacterium* spp., and *Bradyrhizobium* spp. (28), and has appeared as a key enzyme in the biodegradation of many aromatic substances in nature. Extensive kinetic and spectroscopic studies have contributed to elucidation of the mechanism of protocatechuate intradiol dioxygenation by PCase (2, 4, 12, 20–22, 29, 30, 33, 35, 36).

All known native forms of PCase contain equal numbers of two dissimilar α and β subunit types with molecular masses of ca. 22 and 26 kilodaltons (kDa), respectively. One equivalent of ferric iron is usually found in association with each αβ pair. An $(\alpha\beta)_2$Fe association has been reported in the case of *P. aeruginosa* (38), but the usual αβFe type was later found in the same organism (24). However, PCase seem to differ widely in molecular weight according to species: 150,000 in *N. erythropolis* (17), 198,000 in *P. cepacia* (19), 420,000 or 200,000 in *P. putida* (3, 26), and about 700,000 (11, 12, 38) or 586,980 (24) in *P. aeruginosa*. Therefore, all PCase molecules seem to be built of αβ or $(\alpha\beta)_2$ building blocks in various numbers according to species.

Here we report the case of a *Moraxella* sp. that is able to make two different forms of PCase according to the growth substrate. These two varieties had different sizes. According to the data, it is proposed that this bacterial strain synthesized two different but very closely related sets of α and β subunits of PCase, one being made during growth on protocatechuate, the other during growth on guaiacol and several aromatic carbon sources.

MATERIALS AND METHODS

Bacterial growth and crude extracts. The organism used in this study was *Moraxella* sp. strain GU2, the properties of which have been reported (8, 32). It is an oxidase-positive, urease-positive, and encapsulated form that is able to multiply in simple mineral medium containing guaiacol as the sole carbon source. Various aromatic compounds can also support growth of GU2, including 2-ethoxyphenol, anisate, benzoate, catechol, 4-hydroxybenzoate, phenol, protocatechuate, and vanillate. Most phenolic compounds were purchased from Fluka (Buchs, Switzerland), and guaiacol was from Sigma. The various substrates in concentrate solutions were sterilized by filtration and used in the growth medium at 0.6 g/liter (32), with the exception of catechol and phenol (0.1 g/liter). The bacterial cells were grown at pH 6.7 to 7.0 in 10- to 18-liter batches with aeration. Cells were harvested at the end of the exponential growth phase by tangential filtration through a 0.45-μm-pore-size Durapore cassette (Millipore Corp.), followed by a 15-min centrifugation at 18,000 × *g* and 4°C. They were suspended in 20 mM potassium phosphate buffer (pH 7.5) and centrifuged at 45,000 × *g* for 10 min, and the pellet was stored at −20°C before use. The cell paste was diluted with 20 mM potassium phosphate buffer (pH 7.5)–98 μM Mg-EDTA–2% glycerol–5 mM 2-mercaptoethanol. An approximate 1:1 mass ratio of cell paste to buffer was used, and phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor (Sigma), was added up to 0.3 mM in a small volume of ethanol. The thick cell suspension was sonicated at 20 kHz in no more than 30-ml portions for six periods of 1 min each in an ice bath with a Sonimasse instrument (Annemasse, France). Unbroken cells and debris were removed by 10 min of centrifugation at

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45,000 × *g*, and the supernatant was ultracentrifuged for 1 h at 100,000 × *g*. The supernatant thus obtained is designated the soluble extract.

Enzyme assays. The conversion of protocatechuate to β-carboxymuconate was assayed spectrophotometrically (25) in 1 ml of the following mixture: 20 mM potassium phosphate (pH 7.5), 5 mM 2-mercaptoethanol, 3.9 mM protocatechuate (Sigma). Following addition of the enzyme preparation, the decrease in A_{290} was monitored. Taking into account the absorbance of β-carboxymuconate at 290 nm, the molar absorption used for calculations was 2,895 M^{-1} cm^{-1}. At earlier stages of purification, however, β-carboxymuconate was rapidly converted to non-290-nm-absorbing material, and the molar absorption 4,750 M^{-1} cm^{-1} was used instead for calculations. This occurred before the DEAE-Sephadex A-50 column chromatography step in the case of PCase from guaiacol-grown bacteria (PCase-G) and before the hydroxyapatite step in the case of PCase from bacteria grown on protocatechuate (PCase-P), as described in a later section. The amount of enzyme catalyzing the oxidation of 1 μmol of substrate per min at 30°C and pH 7.5 was defined as 1 U of activity.

Catechol 1,2-dioxygenase was assayed by the increase in absorbance at 260 nm (14). Oxidation of other substrates was measured as described before (3) with the following molar extinction coefficients (per molar per centimeter): catechol, 16,900 at 260 nm; gallate, 7,955 at 258 nm; and 3,4-dihydroxyphenylacetate, 9,300 at 260 nm.

Standard buffers. In addition to 98 μM Mg-EDTA and 5 mM 2-mercaptoethanol, the following pH 7.5 buffers contained: buffer P1, 5 mM potassium phosphate, 2% glycerol, and 0.3 mM PMSF; buffer P2, 20 mM potassium phosphate, 2% glycerol, 0.3 mM PMSF; buffer P3, 20 mM potassium phosphate buffer, 5% glycerol, 0.05 to 0.1 mM PMSF.

Purification of PCase from guaiacol-grown cells (PCase-G). The soluble extract was active in the dioxygenation of both protocatechuate and catechol. The solution was brought to 45% saturation successively with powdered ammonium sulfate. After 30 min at 4°C, the protein precipitate was removed by 20 min of centrifugation at 30,000 × *g*. The supernatant was brought to 60% saturation, and the precipitate was collected as above and dissolved in 20 ml of buffer P2.

The protein solution was applied to a hydroxyapatite column (2.5 by 17 cm, Bio-Gel HTP, Bio-Rad Laboratories) equilibrated with buffer P1, and elution was done by a linear 5 to 150 mM linear gradient of potassium phosphate (pH 7.5) in the presence of 98 μM Mg-EDTA, 5 mM 2-mercaptoethanol, 0.3 mM PMSF, and 2% glycerol. Fractions (5 ml) were collected at a flow rate of 50 ml/h. Catechol 1,2-dioxygenase was eluted first and purified separately as described below. PCase appeared from the column at a higher phosphate concentration, and fractions with PCase activity were pooled for further purification. The PCase solution was heated with stirring in a hot-water bath until its temperature reached 50°C, incubated at 50°C for 15 min, and rapidly cooled in ice. Insoluble material was eliminated by ultracentrifugation for 1 h at 100,000 × *g*. Soluble protein was concentrated by ammonium sulfate precipitation, dissolved in P3, and dialyzed overnight against this buffer.

The enzyme solution was applied to a DEAE-Sephadex A-50 column (1 by 3.2 cm) equilibrated with P3 containing 0.1 M NaCl. The column was washed with about 15 ml of P3 containing 0.15 M NaCl, and adsorbed proteins were eluted by a 30-ml linear NaCl gradient from 0.15 to 0.45 M. Fractions with PCase activity were pooled and concentrated to 1 ml by ultrafiltration through an Amicon PM10 membrane. The solution was dialyzed against P3 containing an elevated amount of glycerol (10%), and stored frozen at −70°C.

Purification of PCase from protocatechuate-grown cells (PCase-P). The soluble extract from cells grown on protocatechuate was essentially devoid of catechol 1,2-dioxygenase activity. The crude extract was applied to a DEAE-cellulose DE-52 column (5 by 6 cm) equilibrated with buffer P2. The column was washed with 340 ml of this buffer, and PCase was eluted with 1 liter of P2 containing a linear NaCl gradient from 0 to 0.5 M. Fractions with PCase activity were pooled, and protein precipitation by ammonium sulfate was carried out as described in the preceding section, the final salt concentration being at 65% saturation. The precipitate was collected, dissolved in buffer P2, directly applied to a hydroxyapatite column (2.5 by 15 cm, Bio-Gel HTP as before) equilibrated with P1, and eluted as in the preceding section for purification of PCase-G. Fractions with PCase activity were pooled and applied to a DEAE-Sephadex A-50 column (1 by 2 cm) previously equilibrated with buffer P3. The column was washed with 100 ml of P3 containing 75 mM NaCl, and PCase was eluted by increasing the NaCl concentration to 450 mM in P3.

The enzyme solution (3.6 ml) was applied to a Bio-Gel A-1.5m column (1.5 by 97 cm) equilibrated with P3, and elution was carried out with the same buffer. Fractions (2.5 ml) with PCase activity were pooled and concentrated to 1 ml by ultrafiltration through an Amicon PM10 membrane. The solution was dialyzed against P3 containing an elevated amount of glycerol (10%) and stored frozen at −70°C.

Purification of catechol 1,2-dioxygenase. Catechol 1,2-dioxygenase from the guaiacol-grown bacteria was separated from PCase-G after hydroxyapatite chromatography as indicated above and further purified with a DEAE-Sephadex A-50 column. Active fractions were pooled, concentrated to a small volume, and chromatographed through an Ultrogel AcA 44 column. The purified dioxygenase was virtually homogeneous on the basis of two-dimensional polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of protein samples was carried out in 12% acrylamide gels by the method of Davis (9), slightly modified as follows. The cathodic buffer solution was a mixture of 90 mM glycine and 20 mM Tris hydrochloride (pH 8.4), and the anodic buffer solution was 120 mM Tris hydrochloride (pH 8.4).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by the method of Laemmli (18) with 10% acrylamide and 0.1% SDS in 0.375 mM Tris hydrochloride buffer (pH 6.8), with bromphenol blue as the tracking dye. The stacking gel was 6% acrylamide with 0.1% SDS in 0.125 M Tris buffer, pH 6.8. In procedure A, protein samples were precipitated by trichloracetic acid, then dissolved in 0.125 M Tris buffer containing 1% SDS, 1% 2-mercaptoethanol, and 26% glycerol, and heated for 1 min in a boiling-water bath prior to electrophoresis. Runs were carried out at room temperature at a constant voltage (150 V). In procedure B, protein samples were treated for 1 h at 37°C by 20 mM iodoacetamide in a urea solution (4 M urea, 1% SDS, 0.1 M Tris hydrochloride [pH 8]). In procedure C, protein samples were first reduced for 1 h at 37°C in the urea solution containing 0.14 M 2-mercaptoethanol. Alkylation was performed after addition of iodoacetamide to 0.14 M and a further 20 min of incubation at 37°C. Molecular weight standards were from Pharmacia (Uppsala, Sweden): rabbit

phosphorylase *b* (94,000), bovine serum albumin (67,000), egg ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Standards were treated under the same conditions as the protein samples. Electrophoresis of enzyme in free solution was performed essentially as described before (15). Protein was stained in gels with 0.25% Coomassie blue R250 in 45% methanol–9% glacial acetic acid.

Two-dimensional gel electrophoresis was carried out essentially according to O'Farrell (23). Electrofocusing in the first dimension was done in 4% polyacrylamide gels (0.1 by 9 cm) in the presence of a 7.15% ampholyte mixture (Pharmacia; pH range, 4.5 to 7.5) and 8 M urea. After 18 h and prior to the second dimension run, the gels were soaked for 1 h in 2% SDS–10% glycerol–5% 2-mercaptoethanol–0.06 M Tris hydrochloride, pH 6.8. Separate gels were run for checking the pH gradient after electrofocusing and were sliced into portions 0.5 cm in length. Each gel slice was soaked for 2 h in 1 ml of distilled water, and the pH was determined with a Radiometer GK2421 C combined electrode. Protein markers used in the second dimension were (Pharmacia): bovine milk α-lactalbumin (14,400), soybean trypsin inhibitor (20,100), bovine erythrocyte carbonic anhydrase (30,000), egg white ovalbumin (43,000), bovine serum albumin (67,000), and rabbit muscle phosphorylase *b* (94,000).

Spectrometric analysis. A Uvikon 810 spectrophotometer (Kontron, Velizy, France) was used to gather light absorption data. Electron paramagnetic resonance (EPR) spectra were recorded with an X-band Varian E 109 spectrometer. Low-temperature studies (5 K) were performed with a liquid helium transfer system (ESR 900 system; Oxford Instruments). Standard EPR quartz tubes used were from Wilmard Glass Co. and were filled with 200 μl of the aqueous protein solution.

Purification of denatured PCase subunits. The purified PCase (0.9 to 1 mg) was precipitated either with 10% trichloracetic acid at 4°C or by standing overnight at −20°C in the presence of 4 volumes of acetone. After centrifugation in Eppendorf tubes (20 min, 12,000 × *g*), the precipitate was dissolved with 130 μl of 10 mM Tris hydrochloride (pH 7.6)–150 mM LiCl–6 M urea–0.4 M 2-mercaptoethanol.

Separation of PCase subunits by high-pressure liquid chromatography (HPLC) was done with a 30-nm-pore-size C3 silica column (4.6 by 75 mm; Ultrapore RPSC; Beckman) and a continuous gradient of acetonitrile in 0.1% trifluoroacetic acid. A 50-μl amount of the protein solution was injected at one time. Eluted protein was detected by UV absorbance at 214 nm. The fractions were collected at a flow rate of 0.5 ml/min and lyophilized, and their purity was checked further by SDS-polyacrylamide gel electrophoresis.

Other analytic methods. Protein molecular weights were determined at 4°C by ascending gel filtration on a Sephadex G-200 column (1.5 by 51 cm). Protein samples (0.7 to 2.8 mg) in 0.6 ml of 20 mM potassium buffer (pH 7.5) were eluted with the same buffer containing 10 mM 2-mercaptoethanol at a flow rate of 5.4 ml/h. Protein standards were horse spleen ferritin (450,000), *Bacillus subtilis* L-alanine dehydrogenase (228,000), rabbit muscle aldolase (158,000), hog muscle lactate dehydrogenase (140,000), calf intestine alkaline phosphatase (100,000), bovine serum albumin (68,000), egg ovalbumin (45,000), and bovine chymotrypsinogen A (25,000).

The determinant of iron in PCase was done with the help of the Service Central d'Analyse, Centre National de la Recherche Scientifique, 69390 Vernaison, France. The purified enzyme samples (320 μg of protein) were dialyzed extensively against a blank buffer (20 mM Tris hydrochloride [pH 7.5]), in glassware cleaned with 12 M HCl and ultrapure water (Milli-Q Water Purification System; Millipore).

Edman N-terminal sequencing was also performed at the Service Central d'Analyse by means of a gas-phase automated sequencer (Applied Biosystems 470A) coupled with an HPLC identifier. Phenylthiohydantoin amino acid derivatives were separated with a Spheri-5 column.

Amino acid analysis was done with a PICO-TAG apparatus (Waters Associates) by HPLC chromatography, with the kind help of G. Arlaud (Laboratoire d'Immunologie, Centre d'Études Nucléaires de Grenoble). Protein hydrolysis was performed at 110°C for 24 h with 6 M HCl and 1% (wt/vol) phenol in the absence of oxygen, and amino acids were analyzed after derivation by the phenylisothiocyanate method.

Protein concentrations were estimated by the Bradford method (1), with bovine serum albumin as the standard.

RESULTS

Induction of protocatechuate dioxygenase by guaiacol. Soluble extracts from protocatechuate-grown GU2 cells contained a potent PCase. The enzyme was also present in soluble extracts from guaiacol-grown GU2 cells, together with catechol 1,2-dioxygenase (8). The product of protocatechuate oxygenation by PCase, β-carboxy-*cis*,*cis*-muconate, was identified by the method of Ornston (25) and was accumulated from protocatechuate in the presence of PCase purified as described in Materials and Methods. Both PCase and catechol 1,2-dioxygenase activities were repressed during growth on glucose or succinate.

Gel permeation chromatography through Sephadex G-200 revealed that the soluble PCase of guaiacol-grown cells was almost similar in size to hog lactate dehydrogenase (M_r 140,000) used as a molecular weight standard. In contrast, PCase from protocatechuate-grown cells migrated faster, as a protein with an apparent molecular weight of more than 200,000 by comparison with known standards. This discrepancy also occurred in a chemically different gel matrix, Ultrogel AcA 34 (Fig. 1a and b). The column was 1.5 by 77 cm in size, and the elution buffer used (volume of fractions, 1.8 ml) was 20 mM potassium phosphate (pH 7.5)–100 mM NaCl–10 mM 2-mercaptoethanol.

In order to rule out possible artifacts of gel chromatography and protein modification in extracts, two separate cell batches were prepared from guaiacol and protocatechuate medium and mixed together. A unique soluble extract was made from this bacterial mixture and chromatographed in the same gel column as before. Two PCase fractions with different migration rates were clearly resolved (Fig. 1c). In a variant procedure, extracts from the two cell populations were prepared separately, then pooled, and chromatographed as a mixture on the Ultrogel AcA 34 column. Again two fractions were resolved, with the same distribution as in Fig. 1c. Identical results were obtained with a Sephadex G-200 column instead of AcA 34 in the same conditions. Therefore, the occurrence of two PCase varieties was not explained either by some reacting agent present in one extract and not in the other or by artifactual interaction between protein and the gel matrix. Finally, the shift from production of one type of PCase to the other appeared to operate both ways, just by changing the growth substrate in the culture medium.

The apparent molecular weights of the PCase from guaiacol-grown bacteria (PCase-G) and from cells grown on

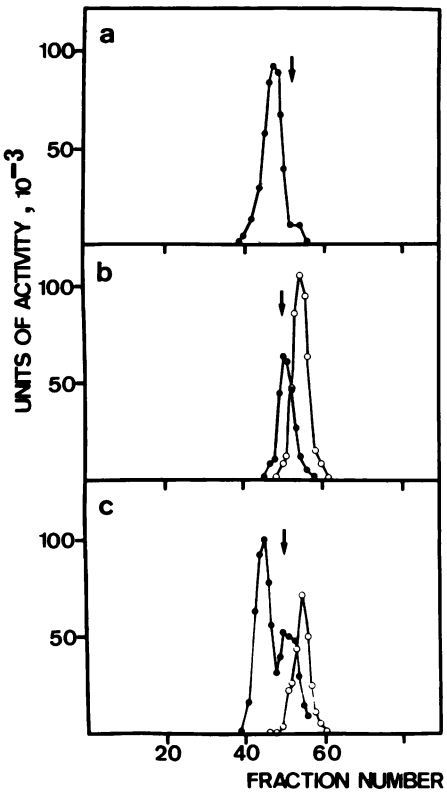

FIG. 1. Ultrogel AcA 34 chromatography of PCases P and G. The arrow indicates the position of the elution peak of hog muscle lactate dehydrogenase used as a marker. Symbols: ●, activity of protocatechuate 3,4-dioxygenase; ○, activity of catechol 1,2-dioxygenase. (a) Soluble extract of protocatechuate-grown cells. (b) Soluble extract of guaiacol-grown cells. (c) Soluble extract prepared from a mixture of two different cell populations, one grown on guaiacol and the other on protocatechuate (see text). Enzyme activities are per milliliter (PCase) or per half-milliliter (catechol dioxygenase).

protocatechuate (PCase-P) were estimated by the gel filtration technique, by comparison with proteins of known molecular weights. The results were 158,000 and 220,000 (± 10,000) for PCases G and P, respectively. It must be noted that PCase activity was also found in GU2 growing on other carbon sources, such as vanillate, 2-ethoxyphenol, 4-hydroxybenzoate, and phenol. In these various instances a dioxygenase was present that behaved like PCase-G through the Sephadex G-200 step. Furthermore, when cells were grown on protocatechuate together with another carbon source such as guaiacol, gel filtration of the extract revealed that only one dioxygenase fraction of the PCase-G type was present.

Purification of the dioxygenases. PCases G and P were purified by separate schemes, as described in Materials and Methods, and the results are summarized in Tables 1 and 2. The specific activity of PCase-G in the crude soluble extract was about twofold higher than that of PCase-P. Hydroxyapatite chromatography was most useful for the purification of both enzymes; in the case of PCase-G, it allowed the complete removal of catechol 1,2-dioxygenase (Fig. 2), an enzyme that was virtually absent in bacteria cultivated on protocatechuate.

TABLE 1. Purification of PCase-G

Fraction	Volume (ml)	Activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)
Soluble extract	100	69	660	0.104	100
$(NH_4)_2SO_4$ precipitate	20	39	320	0.122	57
Hydroxyapatite	47	46	16	2.9	67
Heating	35	29.5	7.2	4.1	43
DEAE A-50	8.8	16.6	1.4	12.9	24

TABLE 2. Purification of PCase-P

Fraction	Volume (ml)	Activity (U)	Protein (mg)	Sp act (U/mg)	Yield (%)
Soluble extract	120	50.1	1,128	0.044	100
DEAE 52-cellulose	90	42.8	306	0.14	85
$(NH_4)_2SO_4$ precipitate	11	31.2	158	0.19	62
Hydroxyapatite	32	27.6	9.0	3.1	55
DEAE Sephadex	4.2	10.7	3.8	2.8	21
Bio-Gel A-1.5m	12.5	9.0	1.37	6.56	18

Both PCases G and P were purified more than 100-fold. Each of the native enzymes appeared as one major protein band after electrophoresis on polyacrylamide gels (Fig. 3). They both displayed the EPR spectrum typical of high-spin ferric proteins with a near-isotropic signal 1.9 mT in width at $g = 4.28$, as already reported (3, 12, 34, 37). Figure 4 shows the result obtained with PCase-P alone after the mixture was flushed for 8 min with oxygen-free argon and addition of 10 mM substrate and then after admission of air to the previous system. Identical spectra were produced from PCase-G by the same protocol.

Subunit composition. Electrophoretic patterns of both enzymes after SDS denaturation were found to be identical, each with equal amounts of two major polypeptides of 25,500 and 29,500 (±1,000) molecular weight (Fig. 3). Therefore, PCases G and P appeared to have the typical $(\alpha\beta)_n$ structure of protocatechuate 3,4-dioxygenase (3, 6, 10, 11, 13, 17, 19, 26, 34), possibly $(\alpha\beta)_3$ and $(\alpha\beta)_4$ for PCase-G and PCase-P, respectively. Minor slow-migrating bands were also repeatedly visible in the SDS gels and were aggregates of α or β chains. It was apparent from electrophoretic data that PCase-G and PCase-P did have similar, if not identical, subunit pairs, despite the fact that the two native enzymes seemed to differ in their molecular sizes. Catechol 1,2-

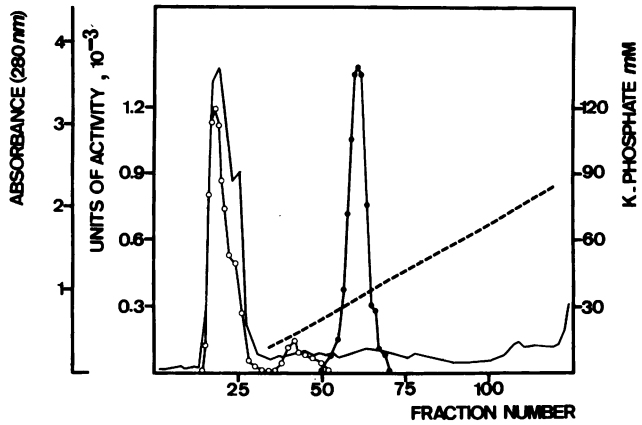

FIG. 2. Separation of PCase-G and catechol 1,2-dioxygenase by chromatography on hydroxyapatite. Conditions as described in the text. Symbols: ——, absorbance of the fractions at 280 nm; ○, activity of catechol 1,2-dioxygenase; ●, activity of protocatechuate 3,4-dioxygenase; – – –, potassium phosphate concentration.

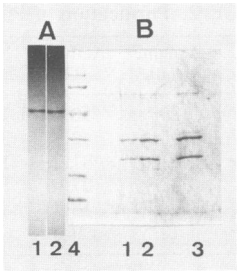

FIG. 3. Polyacrylamide gel electrophoresis of PCases. (A) Native state; (B) denatured with SDS. Track numbers: 1, PCase-P; 2, PCase-G; 3, mixture of PCases G and P; 4, protein standards as specified in the text.

dioxygenase could not be mistaken for PCase in the extract of guaiacol-grown cells because it appeared as a completely separate enzyme that behaved in AcA 34 gel as a protein 68,000 molecular weight. It was characterized and assayed as reported previously (14, 25). It was active on catechol (K_m = 7 μM) and 4-methylcatechol (K_m = 7.5 μM), but it was found to lack any detectable activity on protocatechuate, gallate, and homoprotocatechuate. Gel electrophoresis of the protein in the presence of SDS indicated that it was a dimer of equal subunits, each having a molecular weight of about 35,000.

Pure α and β chains were obtained from both PCases by means of HPLC. The two protein fractions eluted at 48 to 49% acetonitrile. The β subunits eluted ahead of the α subunits (Fig. 5). Both dioxygenases were compared by this technique. The elution diagram obtained from a 1:1 mixture of PCases P and G was identical to the result shown in Fig. 5, indicating that if some differences did exist between the

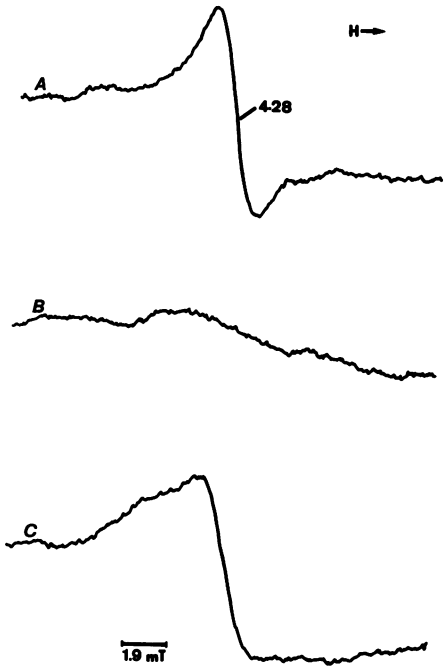

FIG. 4. EPR spectrum of PCase-P. Temperature, 5 K; resonance frequency, 9.25 GHz; power, 30 mW. The enzyme (0.128 mg) was used in 200 μl. Identical spectra were obtained in the same conditions with PCase-G and catechol 1,2-dioxygenase.

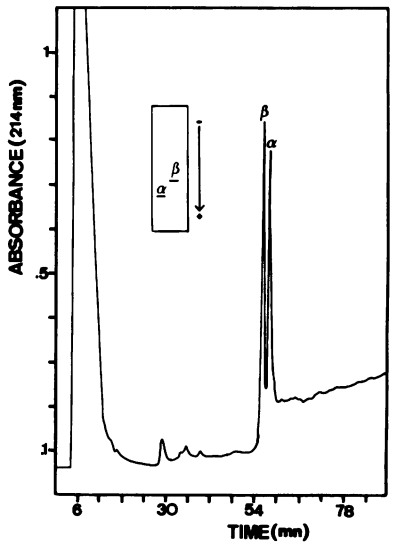

FIG. 5. Resolution of PCase-P subunits by reverse-phase HPLC. PCase-P was purified as described in Materials and Methods. The pure α and β subunits were identified by SDS-polyacrylamide gel electrophoresis (inset). The result obtained with PCase-G was identical.

dioxygenases at the level of their α and β chains, they were so small as to remain unresolved by this technique.

The HPLC technique allowed us to prepare highly purified α and β chains from PCase. The pure α chains of both PCases P and G were compared by N-terminal sequencing as indicated above. Some difficulties were met for the N-terminal determination, possibly because a large proportion of the polypeptides used for sequencing had a blocked N-terminus. With residues which could not be determined with certainty designated by X, the results were the following:

PCase-P(α): X-Asp-Arg-Thr-Ala-Lys-Pro-Ala-Pro-Thr-Tyr-Pro-Val
PCase-G(α): X- X -Arg-Thr-Ala-Lys-Pro-Ala-Pro-Thr-Tyr

Therefore, both α subunits seemed to have the same sequence at their N termini that was different from the corresponding sequence in the PCase of *P. aeruginosa* (16, 38).

Iron content and absorption spectra. The iron contents of purified PCases P and G were estimated. The background value due to metal impurities in the solvent contributed less than 5% of the total for both protein samples. The results for PCases P and G were 2.1 and 1.56 iron atoms per mol of protein, respectively. They are minimal values in the view of possible losses of metal in the course of sample preparation. As a matter of fact they are about half the expected amounts, i.e., 4 and 3 iron atoms per mol for PCases P and G, respectively, considering the ratio of 1 iron per α-β pair that has already been obtained for other protocatechuate dioxygenases (3, 10, 34).

In the visible light range, PCases P and G exhibited a broad absorption band between 400 and 600 nm with a maximum at 428 nm for PCase P (ε = 25,200 M^{-1} cm^{-1}) and 408 nm for PCase G (ε = 15,300 M^{-1} cm^{-1}).

Structural differences. All the data reported above suggested that both PCases P and G had the same α and β subunits in a 1:1 ratio. This conclusion, however, was not supported by higher-resolution electrophoretic conditions or by amino acid composition studies. PCase-G and PCase-P appeared as distinct proteins after two-dimensional electro-

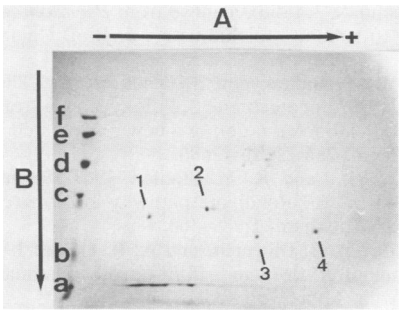

FIG. 6. Two-dimensional polyacrylamide gel electrophoresis of the mixture of PCases P and G. (A) Electrophoresis in the first dimension, pH range from 4.5 to 7.5. (B) Second-dimension separation. Spots 1 and 2, subunit β of PCases P and G, respectively. Spots 3 and 4, subunit α of PCases P and G, respectively. Spots a to f, control protein markers used in the second dimension only, as specified in the text.

phoresis. Both denatured enzymes were examined either separately or as a mixture, and it became evident that the α and β chains of PCase-P were not identical to the α and β chains of PCase-G (Fig. 6). The differences were essentially at the isoelectric pH level. The pI values were 5.95 and 4.80 for the α chains of PCases P and G, respectively, and 7.20 and 6.75 for the β chains, respectively. The two native enzymes also differed in their isoelectric pH, 5.10 for PCase-P and 4.75 for PCase-G.

The occurrence of different α and β chains in PCases P and G was also supported by amino acid composition data. The polypeptides used for analysis were previously highly purified by the HPLC method and examined in identical conditions. The results in Table 3 are average values from three determinations (α and β chains of PCase-P) or two determinations (α and β chains of PCase-G). The aspartate and asparagine content of α and β subunits was significantly higher in PCase-P than in PCase-G. The differences observed for other amino acids between β chains were close to or within experimental error. Consistent other differences were revealed between α chains, however. The α chains of PCase-P were clearly richer in proline and threonine. They were poorer in serine and possibly in the sum of glutamate plus glutamine compared with PCase-G. Conversely, there was no real significant difference between PCases P and G subunits at the level of hydrophobic and cationic residues.

TABLE 3. Partial amino acid composition of PCase-P and PCase-G

Amino acid	No. of residues/mol			
	PCase-G (α)	PCase-P (α)	PCase-G (β)	PCase-P (β)
Alanine	22	25	25	21
Arginine	11	13	18	18
Aspartate + asparagine	9	18	10	23
Glutamate + glutamine	23	16	23	28
Glycine	33	25	32	29
Histidine	6	5	10	9
Isoleucine	6	6	7	6
Leucine	13	15	21	20
Lysine	5	4	6	5
Methionine	4	3	6	5
Phenylalanine	7	10	16	15
Proline	15	26	23	28
Serine	28	10	14	9
Threonine	16	24	18	17
Tyrosine	4	5	8	7
Valine	14	16	18	15

TABLE 4. Substrate specificities of PCases G and P[a]

Substrate	Relative activity (% of control)	
	PCase-G	PCase-P
Protocatechuate (control)	100	100
Catechol	1.8	1.6
Gallate	1.7	0.65
Homoprotocatechuate	2.1	2.9

[a] Assays done at 30°C with the same amount of purified enzyme in 50 mM potassium phosphate buffer (pH 7.5). Substrates were used at 0.1 mM, with the exception of protocatechuate (0.2 mM).

Kinetic data. Both PCases G and P were found to be alike by their specificities and kinetic properties. The K_m of PCase-G for protocatechuate was 11 μM, with a turnover equal to 34 s^{-1}. These values were 22 μM and 24 s^{-1}, respectively, in the case of PCase-P. Therefore, the PCase from guaiacol-grown cells appeared to be a slightly more efficient dioxygenase than its counterpart in protocatechuate-grown bacteria. Table 4 shows that PCases G and P displayed a low level of activity for catechol, gallate (3,4,5-trihydroxybenzoate), and homoprotocatechuate (3,4-dihydroxyphenyl acetate). PCase-G was slightly more active toward gallate than PCase-P. Gallate inhibited protocatechuate dioxygenation by enzymes G and P, with apparent K_is as low as 1 and 0.6 μM, respectively.

DISCUSSION

Induction of PCase activity was observed in *Moraxella* sp. strain GU2 during growth on either guaiacol or protocatechuate and a number of carbon sources, such as 2-ethoxyphenol, anisate, benzoate, catechol, 4-hydroxybenzoate, phenol, and vanillate. This is the first reported occurrence of PCase induction when guaiacol or 2-ethoxyphenol is the sole source of carbon and energy.

Whatever the true physiological inducers of PCase activity are, the production of such an enzyme by cells that are metabolizing guaiacol may seem gratuitous. There is evidence that guaiacol is demethylated by a cytochrome P-450 to catechol (8), which is in turn oxidized to *cis,cis*-muconate by the catechol 1,2-dioxygenase that is also found in these cells. Both intradiol dioxygenases against protocatechuate and catechol are classically known to be regulated independently (5, 27). As judged from known pathways, protocatechuate is not expected to be an intermediate in the degradation of guaiacol. On the other hand, GU2 PCase has been shown to catalyze the dioxygenation of catechol at a significant rate and may contribute to elimination of this toxic compound. Such an explanation remains questionable, however, in the view of the low activity of PCase for catechol (Table 4). It is also not clear why a special type of dioxygenase like PCase-G should be produced to serve this function, since PCase-P is also able to oxidize catechol. The real usefulness of two different enzymes in the physiology of *Moraxella* is not known. Besides its activity toward protocatechuate and catechol, PCase-G may help eliminate one or several other unknown substrates that may be related to the metabolism of guaiacol. In the absence of data, this possibility remains only speculative.

PCase induced by protocatechuate (PCase-P) had a larger apparent molecular size than the PCase induced by guaiacol

and several other carbon sources (PCase-G). Both dioxygenases had the essential features that have been described so far for all other bacterial protocatechuate 3,4-dioxygenases: they contained high-spin ferric iron and were built with dissimilar subunits in equal amounts according to the following structures: $(\alpha\beta Fe)_n$ or $(\alpha_2\beta_2 Fe)_n$ (3, 6, 19, 24, 34, 38). Molecular weights from 22,500 to 26,500, and from 25,000 to 40,000 have been reported for the α and β chains, respectively (19, 34). We have described a new HPLC method for the preparation of α and β polypeptides that may help further analysis.

The number of αβ pairs per molecule was estimated to be 4 in some *P. putida* strains (3) and could be as high as 10 to 12 in *P. aeruginosa* (11, 24, 38). PCase-G, described here, appeared to be relatively small (158,000). It was similar in size to the enzyme of *N. erythropolis* (17), and the $(\alpha\beta)_3$ structure might be proposed for it. The larger PCase-P (220,000) could have the $(\alpha\beta)_4$ structure. It must be assumed that differences in the structures of the subunits were responsible for the different types of assembly in the quaternary structures of PCases P and G.

Considering each αβ pair as a functional site (together with 1 or fewer ferric ions), its turnover number per αβ pair would be 11.3 s^{-1} for PCase-G with protocatechuate as the substrate and a little less than 6 s^{-1} for PCase-P. As shown before, the PCase specific activity was about twice as high in guaiacol-grown bacteria as in cells grown on protocatechuate. This must be compared with the turnover number per αβ pair as indicated above. It is suggested accordingly that the ratio of α and β chains to other protein synthesized in vivo remained about unchanged after the bacteria were shifted from guaiacol to protocatechuate or vice versa.

The two forms of PCase described in this paper appeared to be exclusive of each other, since we have not observed any simultaneous synthesis of both PCases G and P in the same culture. For instance, GU2 in the presence of guaiacol and protocatechuate together in the culture medium yielded PCase-G only. The reversible shift from one form to the other within bacterial cells occurred as an all-or-none process when the carbon source was changed. The occurrence of two alternate forms of PCase in this *Moraxella* sp. might be explained by the presence of two different but closely related sets of cistrons coding for α and β chains, one pair being expressed while the other was not. Another possibility was the occurrence of some posttranslational modifications of a common set of α and β chains leading to changes in their net charges. This was not so likely, however, considering the differences in amino acid composition between the two dioxygenases.

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